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In search of genetic constraints limiting the evolution of egg size: Direct and correlated responses to artificial selection on a prenatal maternal effector

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Abstract

Maternal effects are an important force in nature, but the evolutionary dynamics of the traits that cause them are not well understood. Egg size is known to be a key mediator of prenatal maternal effects with an established genetic basis. Contrary to theoretical expectations for fitness-related traits, there is a large amount of additive genetic variation in egg size observed in natural populations. One possible mechanism for the maintenance of this variation is through genetic constraints caused by a shared genetic basis among traits. Here we created replicated, divergent selection lines for maternal egg investment in Japanese quail (*Coturnix japonica*) to quantify the role of genetic constraints in the evolution of egg size. We found that egg size responds rapidly to selection, accompanied by a strong response in all egg components. Initially, we observed a correlated response in body size, but this response declined over time, showing that egg size and body size can evolve independently. Furthermore, no correlated response in fecundity (i.e. the number of eggs laid over a 10 day period) was observed. However, the response to selection was asymmetrical, with egg size plateauing after one generation of selection in the high but not the low investment lines. We attribute this pattern to the presence of genetic asymmetries, caused by directional dominance or unequal allele frequencies. Such asymmetries may contribute to the evolutionary stasis in egg size observed in natural populations, despite a positive association between egg size and fitness.

Keywords: Indirect genetic effects, *Coturnix japonica*, trade-offs, life history evolution, genetic maternal effects, maintenance of genetic variation

Introduction

The environment experienced during development can have a profound effect on survival and reproduction (Henry & Ulijaszek, 1996; Lindström, 1999). Mothers are often in a unique position to influence this early environment, and so can alter the developmental trajectory of their offspring, through a process known as maternal effects (Mousseau & Fox, 1998). Maternal effects arise from the phenotype of the mother acting on the environment of the offspring, and thereby it's phenotype, and can therefore be influenced by both the mother's genes and the environment she experiences (Wolf et al., 1998). The former is of particular interest from an evolutionary perspective because it allows the traits causing maternal effects (hereafter referred to as maternal effectors) to evolve (Wolf et al., 1998). This in turn can greatly accelerate (positive maternal effects) or impede (negative maternal effects) the response to selection of the affected offspring trait (Kirkpatrick & Lande, 1989; Wolf et al., 1998).

In oviparous species, a large amount of attention has focused on egg size and its role as a maternal effector (Bernardo, 1996). In many taxa, egg size is strongly positively associated with offspring growth and survival in early life (McGinley et al., 1987; Fox & Czesak, 2000; Krist, 2011). Furthermore, many studies have shown egg size to be moderately to highly heritable (Christians, 2002; Fox & Czesak, 2000). However, despite being both heritable and associated with fitness, there is little evidence that egg size responds to contemporary selection in wild populations (e.g. Hõrak et al., 1997). This apparent evolutionary stasis, along with high amounts of additive genetic variance, has been observed in many fitness-related traits (Houle, 1992; Merilä et al., 2001). One proposed solution to

66 this paradox is that evolution is constrained by underlying genetic correlations,
67 caused by a shared genetic basis among traits (hereafter referred to as genetic
68 constraints; Merilä et al., 2001; Walsh & Blows, 2009). Consequently, in order to
69 understand the capacity for this maternal effector to evolve in wild populations,
70 it is necessary to understand its genetic relationships with other fitness-related
71 traits.
72
73 Firstly, it is often assumed that larger eggs represent a larger supply of resource
74 for developing offspring (Bernardo, 1996). However, egg size by itself is not
75 necessarily a measure of maternal resource investment; larger eggs could for
76 example contain more water rather than more lipids or proteins (Fischer et al.,
77 2006). It is therefore unclear whether selection on egg size would result in a
78 correlated response in maternal resource investment. Because the positive
79 association between egg size and fitness is likely caused by these resources (e.g.
80 Finkler et al., 1998), egg size would only be expected to respond to selection in
81 the wild if there are positive genetic correlations between egg size and maternal
82 resource investment.
83
84 Secondly, life history theory predicts that an increase in per offspring investment
85 should come at the cost of fecundity (Smith & Fretwell, 1974). The presence of
86 this trade-off may help explain the maintenance of variation in egg size in wild
87 populations. Such a trade-off may occur as a consequence of resource limitation
88 or via an underlying genetic constraint. Whilst many studies have focused on
89 energetic trade-offs, the evidence for a negative genetic correlation between
90 offspring number and per offspring investment is equivocal in many taxa

(Lessells et al., 1989; Bernardo, 1996; Schwarzkopf et al., 1999; Czesak & Fox, 2003; Fischer et al., 2006).

Finally, egg size might simply be a function of body size (Fox & Czesak, 2000). As there is evidence that there are substantial forces constraining the evolution of larger body size (Blanckenhorn, 2000), if a strong genetic correlation between egg size and body size exists, the evolution of egg size would be constrained by the same forces as the evolution of body size. Consequently it is important to estimate the strength of the genetic correlation between egg size and body size to determine if egg size can evolve independently (Czesak & Fox, 2003).

A powerful way to experimentally test the potential for evolutionary change, as well as for possible genetic constraints, is through artificial selection (Conner, 2003). Artificial selection lines for egg size have been previously established in invertebrates (Schwarzkopf et al., 1999; Czesak & Fox, 2003; Fischer et al., 2006), but the approach is still rarely used in vertebrates because of their often long generation times. Here we created replicated, divergent selection lines for relative egg size (i.e. egg size corrected for female body size) in a captive population of a precocial bird, the Japanese quail (*Coturnix japonica*). Based on these lines, we demonstrate that selection on egg size results in a correlated response in maternal resource investment, and that the evolution of egg size is not constrained by genetic correlations with either fecundity or body size. The response to selection, however, was asymmetrical, which is potentially the result of genetic asymmetries. We suggest that such genetic asymmetries may play an important role in mediating dynamics of egg size evolution in the wild.

Methods

Study population and selection lines

This study was conducted using a captive population of Japanese quail maintained at the University of Zurich, Switzerland. Males and females were housed in separate outdoor aviaries; females in a single sex aviary, and males in a mixed sex aviary together with non-experimental females (both 7 x 5.5m). The founder population (generation 0), consisting of 189 birds (91 females and 98 males), was obtained from a commercial quail egg farm located in the south-east of Switzerland, where birds from two different origins were maintained in two separate populations. These populations had been maintained since 1998 at the farm before our experiment began in 2012, and no (intentional) artificial selection had been imposed on the birds during this time. Although no pedigree was available for the founders, large populations were maintained on the farm, and efforts are made to avoid inbreeding, meaning that the starting stock had a large effective population size. To further increase genetic diversity in our study population, we initially crossed birds from the two origins and used these crosses as the starting population for our selection experiment (generation 1). These birds were randomly split into two replicates consisting of 34 and 38 male-female pairs, respectively (see below for details about the pairings).

We then created replicated, divergent selection lines for high and low maternal egg investment, using relative egg size as the selection criterion. We used relative egg size rather than absolute egg size as the selection criterion in order to not simply select on body size, but rather on the investment a female makes in

141 her eggs independent of her size. Relative egg size was determined as the
142 residuals from a regression of egg size (measured in g egg mass) against female
143 body size (i.e. the first principle component of female body mass and tarsus
144 length; PC1 explained 0.571-0.917 % of variation across generations). These
145 residuals were recalculated for each line, replicate and generation. Relative egg
146 size was highly correlated with absolute egg size in all lines, replicates and
147 generations (mean r ± SD, 0.884 ± 0.120, n=14), but, as expected, not with female
148 tarsus length (-0.002 ± 0.143, n=14) or body mass (0.002 ± 0.143, n=14). The
149 correlation between absolute and relative egg size did not change over the
150 course of the experiment (see Supplementary Material S1).
151
152 In generation 1, the ten females with the largest and smallest relative egg sizes
153 were assigned to the high and low investment line, respectively, in both
154 replicates. In each of the subsequent three generations (i.e. generations 2-4), we
155 selected the most extreme ten pairs (50%) in both the high and low lines of both
156 replicates. The eggs of the selected pairs were collected, incubated and hatched
157 as outlined below. Two sons and two daughters from each selected pair were
158 then used for the next breeding round, giving 20 breeding pairs per line,
159 replicate and generation. If the selected pairs did not have enough sons and
160 daughters, we used offspring from the 11th and 12th ranked pairs to make up
161 the total of 20 pairs within a line replicate. Within a replicate, the high and low
162 investment lines were always bred simultaneously to control for seasonal and
163 age effects. In addition, an unselected base population was maintained in the
164 same facility, originating from the same founder populations. These birds were
165 not bred at the same times or ages as the selection lines and so are not directly

166 comparable. Nevertheless, given that there was no directional change in mean
167 egg size over 5 generations in this unselected population ($F_{1,3}$ = 0.51, P = 0.528;
168 see Figure S2), we can exclude the possibility that systematic changes in egg size
169 have occurred over time due to inadvertent effects of husbandry.
170
171 *Breeding protocol*
172 For breeding, males and females were brought into cages (122 x 50 x 50 cm) in
173 our breeding facility for three to four weeks, and body mass and tarsus length
174 were measured (to the nearest 1g and 0.1mm, respectively). Our facility is kept
175 on a 16:8 light:dark cycle at approximately 20°C. Cages contained *ad libitum*
176 food, water, grit, a source of calcium, a house and a raised sand bath. The bottom
177 of the cages was filled with sawdust. We kept one male-female pair per cage.
178 Breeding pairs consisted of non-related individuals from the same line and
179 replicate. Non-related was defined as individuals not sharing any grandparents.
180 This resulted in no quail having an inbreeding coefficient > 0.016 by generation 4
181 (assuming that the initial population consisted of unrelated individuals). All
182 individuals were bred once, with the exception of birds from generation 4, which
183 were let back into the aviary for seven to nine weeks after the first breeding
184 round and then brought back into breeding cages, where they were bred with
185 different partners.
186
187 Eggs were collected every morning over a period of 14 days. They were labeled
188 with a non-toxic marker and weighed (to the nearest 0.01g). The first two eggs
189 were dissected (see below) and the middle eggs (from the sixth and seventh
190 days) were frozen. The remaining eggs were stored at 12°C before being

191 artificially incubated (Favorit, HEKA Brutgeräte, Rietberg, Germany). During the
192 first 14 days eggs were incubated at 37.8°C and 55% humidity. They were then
193 candled and all developed eggs were transferred to a hatcher (Favorit, HEKA
194 Brutgeräte, Rietberg, Germany) in individual compartments, and kept at 37.6°C
195 and 80% humidity until hatching. Hatchlings were marked with an individually
196 numbered plastic leg ring and kept in a heated cage (109 x 57 x 25 cm,
197 Kükenaufzuchtbox Nr 4002/C, HEKA Brutgeräte, Rietberg, Germany) for two
198 weeks. For the first five days the temperature was kept at 35 - 38°C, then slowly
199 lowered to 25°C over the next nine days. After two weeks, the chicks were
200 transferred to cages within our breeding facility. At four weeks of age they were
201 sexed according to their plumage and put into the outdoor aviaries.
202
203 *Laying rate and laying intervals*
204 As quails are indeterminate layers (Cole, 1917), removing an egg every day (as
205 necessary to successfully store and artificially incubate eggs) causes the females
206 to continually lay eggs. Therefore we cannot directly measure clutch size in our
207 system, and we used an indirect measure of fecundity instead: the proportion of
208 days on which a female laid an egg while in her cage (hereafter referred to as
209 laying rate), a measure that is commonly used to quantify fecundity in poultry
210 (e.g. Wright et al., 2012). Females may be constrained in how fast egg nutrients
211 can be deposited, resulting in larger eggs taking longer to produce (e.g. Meijer,
212 1992). Under this scenario, we would predict an increase in the time between
213 two eggs, as well as in the number of laying gaps (Williams, 2012, and references
214 therein) in the high investment line. In our population, most females lay between
215 6 and 10 eggs in a 10-day period (J.L.P, personal observation). This range in

laying rate is equivalent to the variation in clutch size seen in many natural populations (Johnsgard, 1988). For females in generation 4, we furthermore recorded the time between laying two eggs (hereafter referred to as laying interval), during the third week the females were in cages. All cages were checked every hour up until one hour before lights off (21:00), recording the hour in which an egg was found in a cage. This was done three or four days in a row to ensure that two eggs were collected from each female on consecutive days. Eggs found in the morning were assumed to have been laid in the hour before lights off.

In generation 5 we brought fewer females into cages than in previous generations and for a shorter period of time (some for only one week). We therefore did not quantify laying rate in this generation, and the sample size in generation 5 was reduced compared to previous generations.

Egg measurements

Starting from the third generation, one or two eggs were dissected from each female. Wet yolks were weighed and shells were dried at 80°C until a constant weight and weighed (both to nearest 0.001g). Albumen mass was calculated as the total egg mass minus yolk and shell masses. For generation 4, we furthermore separated eggs into yolk, albumen and shell, weighed (wet mass) and dried them in a drying oven at 80°C for a minimum of 15 hours and weighed them again (dry mass). Dry masses of eggs laid on the first two days, as well as on the 15th and 16th days the females were in cages were determined in this generation, to test the consistency of egg composition across the laying

sequence. Egg size and all egg components were highly repeatable within females (Table S1). Differences between eggs laid at the beginning and the end of a laying sequence are described in the Supplementary Material S3.

All procedures were conducted under licenses provided by the Veterinary Office of the Canton of Zurich, Switzerland (permit numbers 195/2010; 14/2014; 156).

Statistical analyses

Response to selection and realized heritability

For the analysis of the response to selection we followed the methods outlined in Falconer & Mackay (1996). Using the breeder's equation

$$R = h^2 S$$

where R is the response to selection, S is the selection differential and h^2 is the narrow-sense heritability, we calculated the realized heritability (h_r^2), defined as the heritability as realized from the response to selection (Falconer & Mackay, 1996). Our experiment ran over multiple generations, which allowed us to estimate h_r^2 and its associated error by regressing the cumulative response to selection (R_C) against the cumulative selection differential (S_C) and forcing the intercept through 0 (the difference between the two lines in the initial population), giving

$$R_C = bS_C$$

where $b = \frac{1}{2}h_r^2$, as selection was only on female phenotypes and we assume autosomal inheritance (Falconer & Mackay, 1996). The cumulative response to selection is a robust way to assess selection response, as it is a means to overcome variation between generations due to for example environmental

fluctuations that can distort the interpretation of per-generation responses to selection (see Falconer & Mackay, 1996, p194-198). *R* was calculated as the mean egg size of the offspring generation minus the mean egg size of the parental generation (egg size is defined here as absolute egg size; relative egg size was calculated within generations and thus is inappropriate for between-generation comparisons). *S* was calculated as the mean egg size of selected mothers minus the mean egg size of the entire parental generation. We corrected for reproductive difference between females (i.e. having different numbers of offspring) by weighting the mean of selected parents by the number of daughters per female in the next generation (effective selection differential; Falconer & Mackay, 1996). Egg size was standardized (mean=0, SD=1, across all data) prior to calculation of *R* and *S*, meaning that *S* is equivalent to the selection intensity on females (i_f) and the selection intensity (i) is equal to $\frac{1}{2} i_f$ (Falconer & Mackay, 1996).

As we bred the quail throughout the year, there were substantial environmental differences between generations and replicates in some measured traits (see Figures 1 and S2). Given that we bred both lines within a replicate at the same time, we can control for these seasonal effects by using the two lines within a replicate as controls for each other (Hill, 1972; Falconer & Mackay, 1996). Therefore R_d and S_d were calculated as the differences in *R* and *S* between the lines within each generation and replicate. R_d and S_d were then used to calculate R_C and S_C. R_C and S_C therefore represent cumulative differences between the lines. We tested for a difference in h_r^2 between the two replicates, through testing

for an interaction between S_C and replicate on R_C. We also calculated an overall h_r^2 by pooling the two replicates.

As h_r^2 was calculated using the differences between the two lines, we could not test directly for differences in h_r^2 between the lines. As we were interested in testing for the presence of an asymmetric response to selection, we also tested for an interaction between line and S_C on R_C, where R_C and S_C were calculated from R and S, including data from both lines and replicates. Replicate was included as a factor in the model. This analysis was performed for generations 2-5, as the mean egg size in generation 1 was clearly different from other generations. As it has been suggested that heritability may not represent the most informative metric of evolutionary potential (Houle, 1992), we also calculated the coefficient of additive genetic variance (CV_A) as

$$CV_A = \frac{\sqrt{h^2 V_P}}{\mu}$$

where μ is the phenotypic mean and V_P is the phenotypic variance, and evolvability (I_A) as ${CV_A}^2$.

Correlated responses to selection and realized co-heritabilities

For wet yolk mass, wet albumen mass, dry shell mass, yolk/albumen ratio, laying rate, female body mass and female tarsus length, we had information over enough generations to calculate the co-heritability of these traits with egg size. Co-heritability ($r_A h_X h_Y$) is the strength to which one trait (Y) responds to selection on another trait (X) (i.e. the correlated response) and is a composite of

the heritability of the two traits and the genetic correlation between them (Falconer & Mackay, 1996). Co-heritability can be calculated from

$$CR_Y = r_A h_X h_Y S_X \frac{\sigma_{PY}}{\sigma_{PX}}$$

where r_A is the genetic correlation between trait X and Y, h is the square root of the heritability of a trait, CR_Y is the correlated response of trait Y to selection on trait X and σ_P is the standard deviation of a trait (derived from Falconer & Mackay, 1996, eqn. 19.6).

As with the realized heritability, given that we had data over multiple generations, we estimated co-heritability between a trait and egg size from the regression of cumulative correlated response in that trait (CR_{CY}) against the cumulative selection differential of egg size (S_{CX}). In order to calculate CR_Y and S_X, we again used the difference between high and low lines within each generation and replicate. For these calculations, we standardized all variables (mean=0, SD=1, across all data), meaning that $\frac{\sigma_{PY}}{\sigma_{PX}} = 1$. Therefore the slope of the regression is equal to the co-heritability and as selection was only on female phenotypes this estimate (and SE) was doubled (Falconer & Mackay, 1996). We tested for a difference in co-heritability between the two replicates, through an interaction between S_{CX} and replicate. We also calculated an overall co-heritability by pooling the data from the two replicates. Furthermore, we tested for a difference in co-heritability among egg traits by testing for an interaction between S_{CX} and trait using pooled data from both replicates. Significance of pairwise differences between these traits was obtained by calculating a t statistic from the estimated difference between slopes. After visual inspection of the data,

we also tested for a non-linear co-heritability for body mass and tarsus length, by including a quadratic S_{CX} term and pooling replicates.

Dry egg components and laying behavior

In generation 4, we additionally tested for differences between the lines in the mass of dry egg components and in laying intervals. We ran linear models including line and replicate as fixed factors. For the dried egg components data, the mean values for each constituent (yolk, albumen, shell and total dry mass) were calculated from all measured eggs of a female. We also ran models to correct for possible allometric relationships of the dry egg constituents with egg size and body size. The response variables were log transformed, and included log transformed body size (tarsus length cubed) or egg size as a covariate. Given that we have selected on relative egg size, we would expect a larger amount of dry components in the high investment line when controlling for body size, but no difference between the lines when controlling for egg size.

For the laying interval data, we calculated a mean value for all females for which we had more than one laying interval. Given that we could not be completely sure that eggs laid after the cages were last checked were laid in the hour before dark, we also ran an analysis excluding any laying intervals that were calculated including that hour. One high line female was excluded from both analyses as her laying interval was > 4 SD out of the range shown by other females.

In addition to the analyses described above, we also present the results of t-tests of difference in all traits between the lines in generation 4 (replicates pooled). All analyses were run in R (3.0.3, R Core Team, 2014).

Results

Response to selection

The selection intensity (i) was 0.344 ± 0.105 (mean ± SD) in the high investment line and -0.201 ± 0.087 in the low investment line. After only one generation of selection, the lines differed significantly in absolute egg size (Figure 1, Table S3). This response increased as the selection experiment progressed as demonstrated by the high h_r^2 (Tables 2, Figure 2a). By generation 4, there was a 1.25 SD difference in absolute egg size between the lines (Tables 1, Figure 1). Although the response to selection was strong in both replicates, it was weaker in the second replicate, with a significantly lower h_r^2 (Table 2, Figure 2a). Using h_r^2 we estimated CV_A as 0.075 (replicate 1: 0.081; replicate 2: 0.071) and I_A as 0.0056 (replicate 1: 0.0066; replicate 2: 0.0050). When comparing the response to selection between the two lines, we found a significant difference (S_C x line: $F_{1,11}$ = 13.37, P = 0.004), with a strong response in the low investment line ($F_{1,5}$ = 41.63, P = 0.001), but no apparent response in the high investment line ($F_{1,5}$ = 0.12 , P = 0.746), after the first generation of selection.

Correlated responses to selection in egg components

All egg components showed a strong and significant correlated response to selection on egg size (Table 2, Figure 2). The differences between lines in generation 4 are shown in Table 1. Overall egg components differed in the

strength of their correlated response to selection on egg size (S_{CX} x trait: $F_{2,21}$ = 7.85, P = 0.003, Figure 2). The co-heritability of albumen mass was significantly higher than that of both yolk mass (t_{21} = 2.28, P = 0.033) and shell mass (t_{21} = 3.95, P < 0.001), but there was no significant difference between yolk mass and shell mass (t_{21} = 1.66, P = 0.111). The correlated response of shell mass did not differ between replicates. However yolk mass responded significantly less in the second replicate and there was a tendency for the same pattern in albumen mass (Table 2). Yolk/albumen ratio did not show a correlated response to selection on egg size (Table 2).

In order to test if these correlated responses of egg components correspond to changes in maternal resource investment, we examined the dry egg constituents. As expected, high line eggs had absolutely more dry constituents than low line eggs (Tables 1 and 3). All dry components had a significant positive relationship with egg size and all but dry yolk mass had a significant positive relationship with body size (Table 3). Eggs from the high investment lines contained more dry components than eggs from the low investment lines when correcting for body size. When correcting for egg size, however, eggs from the high investment lines contained less yolk and more albumen (Table 3) and the two lines did not differ in shell or total dry mass (Table 3). Both dry albumen and yolk masses correlated strongly with wet albumen and yolk masses respectively (albumen: r = 0.938, P < 0.001, N = 80; yolk: r = 0.996, P < 0.001, N = 80).

Correlated response to selection in laying behavior

Laying rate showed no correlated response to selection on egg size, as shown by a non-significant co-heritability (Table 2). Similarly there was no difference between the lines in laying interval ($F_{1,76}$ = 0.770, P = 0.383, Table 1) and no difference between the replicates ($F_{1,76}$ = 0.376, P = 0.542). When excluding laying intervals where one of the laying times was inferred (see Methods) the results were qualitatively similar (line: $F_{1,60}$ = 0.424, P = 0.518; replicate: $F_{1,60}$ = 1.015, P = 0.318).

Correlated response to selection in body size

Both tarsus length and body mass showed a significant non-linear correlated response to selection on egg size, with an initial strong correlated response that declined during the course of the selection experiment (Table 2, Figures 2c and d).

Discussion

We show that maternal egg investment responds rapidly to directional selection, with lines differing in egg size by more than one standard deviation after only four generations of selection. This rapid response of egg size to selection is remarkable given the relatively weak selective pressure that was applied to female phenotypes only, with the mean selection intensity being well within the range seen in natural populations (Kingsolver et al., 2001). Also, selection only on females reflects what would occur in the wild, where selection acts only upon individuals expressing a phenotype. If variation in egg size is associated with variation in fitness, as is commonly found (Krist, 2011), egg size should respond rapidly to selection in wild population.

Although intuitive, it is not inevitable that selection on egg size results in a correlated response in resource investment (Fischer et al., 2006). In our selection experiment, a strong correlated response in yolk, albumen and shell mass was observed. Furthermore, eggs from the high investment lines contained more dry components, which are almost entirely lipids and proteins in yolk, proteins in albumen and calcium in the shell (Romanoff & Romanoff, 1949). All of these resources are vital to the successful development of the chick (Finkler et al., 1998). Producing larger eggs thus constitutes a higher resource investment by the mother.

Eggs from the high maternal investment lines had more dry albumen than eggs from the low maternal investment line, even after controlling for egg size. Furthermore, albumen mass responded more strongly to selection on egg size than did yolk mass. This is in line with previous findings in chickens, where selection on yolk/albumen ratio resulted in a larger response in albumen mass than yolk mass (Miyoshi et al., 1996). Albumen consists of a high proportion (around 50%) of egg protein in precocial species (Carey et al., 1980) and because albumen is the main protein source for the chick during prenatal development (Freeman & Vince, 1974) it is likely a limited factor during critical periods early in life.

Although we here show that egg size can rapidly respond to selection and results in a correlated response in resource investment, evolutionary stasis in egg size is observed in many natural populations. One mechanism that could contribute to

455 this phenomenon is a size/number trade-off, which is a key concept in life
456 history theory (Smith & Fretwell, 1974). To date, however, there is surprisingly
457 little empirical evidence for such a constraint at the genetic level (Bernardo,
458 1996; Schwarzkopf et al., 1999; Czesak & Fox, 2003; Fischer et al., 2006). In wild
459 bird populations, for example, so far only three studies have tested for a genetic
460 correlation between egg size and clutch size (Lessells et al., 1989; Garant et al.,
461 2008; Santure et al., 2013), two of which were performed in the same population
462 (Garant et al., 2008; Santure et al., 2013). The results of these studies were
463 equivocal with only one showing a significant negative genetic correlation
464 (Garant et al., 2008). Similarily, no correlated response in egg size was observed
465 in Japanese quail artificially selected for lay rate (Nestor et al., 1983). In line with
466 these previous findings, we found no differences in laying rate or laying intervals
467 between the two selection lines, suggesting that females of the high and low
468 investment lines produced differently sized eggs in the same amount of time (see
469 also Christians & Williams, 2001). Although we could only use an indirect
470 measure of fecundity in our study, this result suggests that there is not an
471 inevitable genetic trade-off between egg size and egg number. Additional studies
472 in wild bird populations that test for a (lack of a) genetic correlation between egg
473 size and clutch size are, however, required to confirm that the two life history
474 traits can evolve independently under natural conditions.
475
476 Given that we fed our birds *ad libitum*, we cannot rule out the presence of an
477 energetic trade-off when resources are limited (McGinley et al., 1987; Czesak &
478 Fox, 2003), as is the case in most wild populations. However, the presence of an
479 energetic trade-off is also largely unsupported in wild-living birds. For example,

480 manipulations forcing birds to lay additional eggs do not necessarily result in
481 smaller replacement eggs (Nager et al., 2000; Williams & Miller, 2003), and in
482 one study the number of additional eggs laid was even positively correlated with
483 egg size (Williams & Miller, 2003). Hormonal manipulations targeting the
484 potential mechanistic basis of an egg size/number trade-off have also found
485 equivocal results. For example, increasing plasma follicle stimulating hormone
486 (FSH), which is known to mediate the egg number/size trade-off in lizards
487 (Sinervo & Licht, 1991), resulted in a decrease in both egg size and clutch size
488 (Christians & Williams, 2002).
489
490 Our measure of relative egg size was highly phenotypically correlated with
491 absolute egg size, but not with body size. Therefore, at least on a phenotypic
492 level, we were not directly selecting on body size. Nevertheless, we saw a strong
493 initial response in body size, indicating that there was a strong underlying
494 genetic correlation. This initial response mirrors what was seen in a selection
495 experiment in chickens, where both selection on body size and on egg size
496 resulted in a similar magnitude of response in egg size (Festing & Nordskog,
497 1967). Moreover, phenotypic correlations between egg size and body size were
498 found to be much weaker than genetic correlations (Festing & Nordskog, 1967).
499 This suggests that weak phenotypic correlations in the wild may hide potentially
500 strong genetic correlations, which seems to be the case in our population.
501
502 Despite the strength of this initial correlated response in body size, it diminished
503 rapidly as we selected further for relative egg size. This indicates that the genetic
504 correlation between egg size and body size is either not stable, and/or that there

505 is substantial additive genetic variation for relative egg size (Czesak & Fox,
506 2003). Furthermore, the increases in resource investment were not simply a
507 function of larger body size. There was a strong difference between the lines in
508 dry egg components, even after correcting for body size, and dry yolk mass was
509 in fact not related to body size. Our results therefore show that it is indeed
510 possible to select on relative resource investment, contrary to long held beliefs
511 (Hutt, 1949).
512
513 Overall, we found little support for a role of body size or fecundity in
514 constraining the evolution of egg size. We cannot exclude the possibility,
515 however, that other genetic constraints or energetic trade-offs, not assessed in
516 our study, may constrain the evolution of increased maternal investment. These
517 could include trade-offs between reproductive investment and immunity
518 (Knowles et al., 2009), predator escape performance (Cooper et al., 1990; Lee et
519 al., 1996) and/or physiological damage, such as oxidative stress (Monaghan et
520 al., 2009). All of these trade-offs would ultimately act to reduce an individual's
521 lifespan. This has been investigated mostly in the context of total reproductive
522 investment, rather than per offspring investment. Also, results have been
523 somewhat inconclusive, with no overall effect of increased reproductive
524 investment on lifespan being found across studies in birds (Santos & Nakagawa,
525 2012).
526
527 Whereas we found little evidence for genetic constraints limiting the evolution of
528 maternal investment, there was evidence that the response to selection between
529 the two lines was asymmetrical. Between generations 2 and 5 egg size did not

increase in the maternal high investment lines but rapidly decreased in the maternal low investment lines. Interestingly, such asymmetrical responses to selection are regularly observed in fitness-related traits (Frankham, 1990).

There are several possible explanations for this phenomenon. Firstly, as we were selecting for egg size relative to body size, it is possible that we reached a physiological and/or morphological maximum. However, this seems unlikely, as females occasionally lay double-yolked eggs of over 21g in our population (J.L.P personal observation). Secondly, drift, inbreeding depression or different selection pressures can cause asymmetric responses to selection (Frankham, 1990; Falconer & Mackay, 1996). However, we can exclude them all given the design of our experiment. Thirdly, when traits are themselves affected by maternal effects, this can alter their response to selection (Kirkpatrick & Lande, 1989). However these models do not predict the asymmetric responses found in our study. Finally, genetic asymmetry can cause asymmetric responses to selection in fitness-related traits (Frankham, 1990). Genetic asymmetry refers to both directional dominance and unequal allele frequencies (Falconer & Mackay, 1996). The presence of deleterious recessive alleles makes it easy to select downwards, by increasing the frequency of homozygotes for these recessive alleles, but not upwards as the dominant alleles are already expressed in most cases (assuming equal allele frequencies in the base population). In the case of unequal allele frequencies, if the alleles for high fitness are at higher frequency, it similarly becomes difficult to select for higher fitness. Such genetic asymmetries affect the susceptibility of a trait to inbreeding depression, which has, in fact,

been observed for egg size in Japanese quail (Sittmann et al. 1966; see Shoffner 1948; Wang & Pirchner 1992 for chickens).

In conclusion, we found that egg size responds rapidly to selection along with a correlated response in maternal resource investment. Given the positive effects of maternal resource investment on offspring phenotype (e.g. on juvenile size; Krist, 2011), the ability of egg size to evolve will therefore act to accelerate the response to selection of these offspring traits (Kirkpatrick & Lande, 1989; Wolf et al., 1998). We found no evidence for a genetic trade-off between egg size and our indirect measure of fecundity. Furthermore, despite a genetic correlation between egg size and body size, we show that it is possible to successfully select for relative egg size. It is therefore unlikely that selection on body size would constrain the evolution of egg size. Instead, genetic asymmetries may constrain the evolution of larger eggs. Testing for the presence of inbreeding depression in egg size in wild populations, which would provide evidence for such asymmetries, would therefore prove a fruitful next step to understand the mechanisms that shape the evolutionary dynamics of such prenatal maternal effectors.

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580

581 **Conflict of Interest**

582 The authors declare no conflict of interest.

583

584 **Data Archiving**

585 Data are available from Dryad Digital Repository:

586 http://dx.doi.org/10.5061/dryad.gj5b7

587

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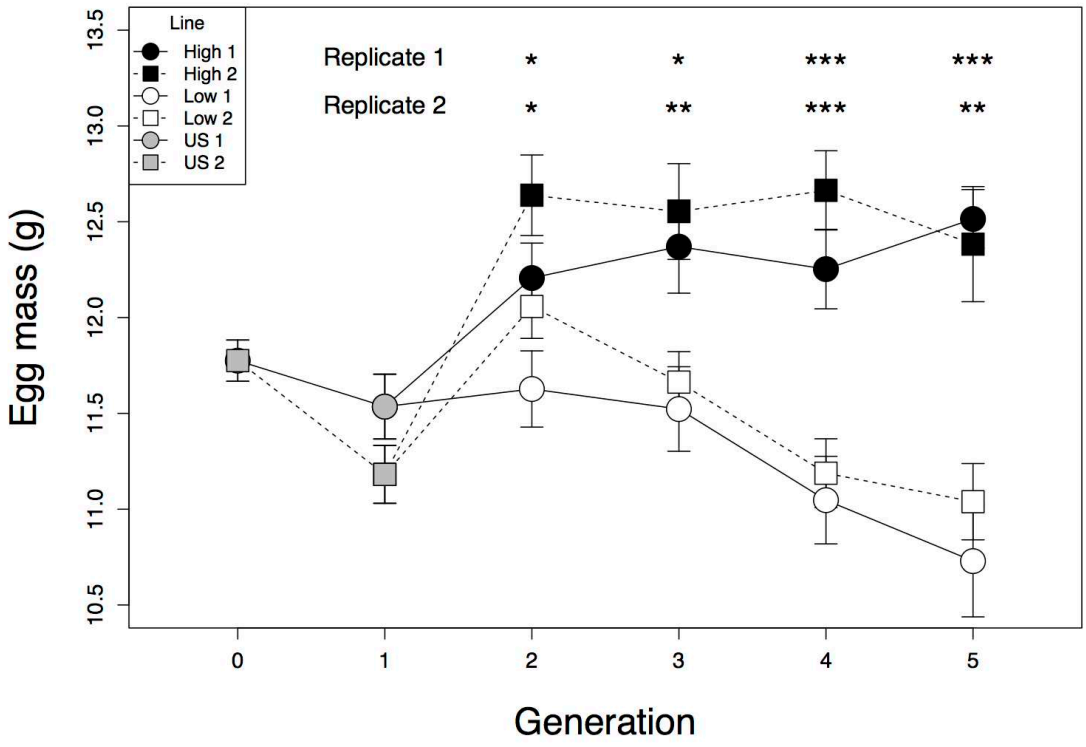
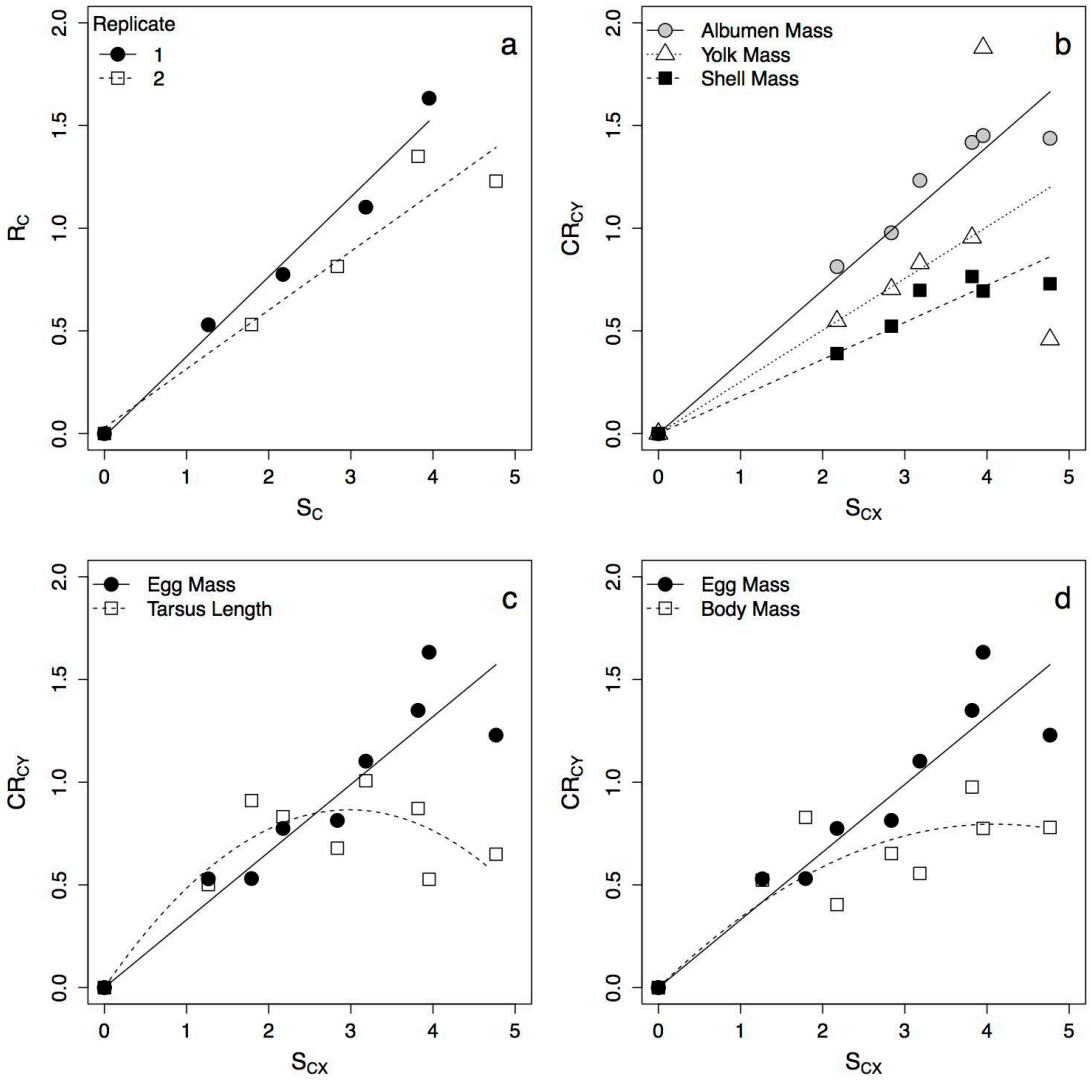
772 **Figures**



773

774 Figure 1: Absolute egg size in the selection lines over the course of 6 generations
775 (Mean±SE). US represents the unselected founder population. Stars represent
776 the difference between high and low maternal investment lines in each replicate
777 and generation (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

778



779

780 Figure 2. Response to selection of a) absolute egg size in the two replicates, b)
781 yolk, albumen and shell mass (replicates pooled), c) absolute egg size and tarsus
782 length and d) absolute egg size and body mass. Both axes are in units of SD. S_{CX} is
783 the cumulative selection differential on absolute egg size between the lines, R_C is
784 the cumulative response to selection in absolute egg size between the lines and
785 CR_{CY} is the cumulative correlated response to selection between the lines in traits
786 indicated by the legends. Lines represent estimates of realized (co-)heritability.

787

788

Tables

Table 1: Average phenotypes and testing for differences between selection lines for high and low maternal egg investment. Mean ± SD of traits measured in generation 4 are presented. Data from the two replicates were pooled. Significant differences are displayed in bold.

Trait	High	Low	*t*	df	*P*
Egg Size					
Egg Size (g)	12.46 ± 0.94	11.12 ± 0.91	**6.49**	**78**	**<0.001**
Dry Mass (g)	3.857 ± 0.334	3.411 ± 0.325	**6.04**	**78**	**<0.001**
Egg Components					
Wet Albumen (g)	7.851 ± 0.570	6.879 ± 0.523	**7.95**	**78**	**<0.001**
Dry Albumen (g)	0.912 ± 0.088	0.789 ± 0.070	**6.96**	**78**	**<0.001**
Wet Yolk (g)	3.620 ± 0.413	3.228 ± 0.365	**4.49**	**78**	**<0.001**
Dry Yolk (g)	1.899 ± 0.229	1.699 ± 0.213	**4.05**	**78**	**<0.001**
Dry Shell (g)	1.044 ± 0.090	0.922 ± 0.077	**6.51**	**78**	**<0.001**
Yolk/Albumen Ratio	0.461 ± 0.042	0.469 ± 0.036	0.87	78	0.387
Fecundity					
Laying Rate	0.915 ± 0.034	0.922 ± 0.044	0.71	78	0.478
Laying Interval (hours)	24.18 ± 0.55	24.07 ± 0.55	0.88	77	0.383
Body Size					
Tarsus Length (mm)	40.6 ± 1.2	39.3 ± 1.4	**4.55**	**78**	**<0.001**
Body Mass (g)	281 ± 24	260 ± 28	**3.57**	**78**	**<0.001**

Table 2: Realized heritability of absolute egg size and co-heritabilities of different traits with absolute egg size (Estimate ± SE). Boldface numbers indicate significant estimates. Difference refers to the interaction between replicate and selection differential. For body size, we estimated non-linear co-heritabilities and so a and b represent estimates from $Y = aX + bX^2$

	Pooled				Replicate 1	Replicate 2	Difference		
	Estimate	*F*	df	*P*			*F*	df	*P*
Realized Heritability									
Absolute Egg Size	**0.660 ± 0.038**	290.83	1,9	<0.001	**0.768 ± 0.042**	**0.588 ± 0.034**	10.92	1,8	0.011
Egg Components									
Wet Albumen Mass	**0.698 ± 0.024**	793.73	1,7	<0.001	**0.750 ± 0.032**	**0.664 ± 0.026**	4.3	1,6	0.084
Wet Yolk Mass	**0.504 ± 0.100**	25.23	1,7	0.002	**0.738 ± 0.118**	**0.346 ± 0.096**	6.77	1,6	0.041
Dry Shell Mass	**0.362 ± 0.018**	448.65	1,7	<0.001	**0.382 ± 0.028**	**0.348 ± 0.022**	0.93	1,6	0.371
Yolk/Albumen Ratio	-0.072 ± 0.108	0.45	1,7	0.526	0.162 ± 0.136	-0.230 ± 0.112	4.91	1,6	0.069
Fecundity									
Laying Rate	-0.056 ± 0.038	2.26	1,7	0.177	-0.066 ± 0.064	-0.050 ± 0.052	0.04	1,6	0.85
Body Size									
Female Tarsus Length	**a = 0.581 ± 0.068** **b = -0.097 ± 0.018**	106.4	2,8	<0.001					
Female Body Mass	**a = 0.389 ± 0.077** **b = -0.047 ± 0.02**	69.64	2,8	<0.001					

Table 3: Differences in dry egg components between selection lines. For each egg component we ran three analyses, one without covariates (a), one controlling for body size (b) and one controlling for absolute egg size (c). Data from generation 4 are shown. Significant effects are displayed in bold.

Trait	Model	Line			Repeat			Tarsus			Egg Mass		
		F	df	*P*	*F*	df	*P*	*F*	df	*P*	*F*	df	*P*
Dry Albumen													
	a	**48.2**	**1,77**	**<0.001**	0.58	1,77	0.45	-	-	-	-	-	-
	b	**27.97**	**1,76**	**<0.001**	1.24	1,76	0.268	**5.28**	**1,76**	**0.024**	-	-	-
	c	**4.84**	**1,76**	**0.031**	0.2	1,76	0.655	-	-	-	**125.81**	**1,76**	**<0.001**
Dry Yolk													
	a	**17.15**	**1,77**	**<0.001**	**4.53**	**1,77**	**0.036**	-	-	-	-	-	-
	b	**7.82**	**1,76**	**0.007**	**5.12**	**1,76**	**0.026**	2.57	1,76	0.113	-	-	-
	c	**8.08**	**1,76**	**0.006**	3.02	1,76	0.086	-	-	-	**223.04**	**1,76**	**<0.001**
Dry Shell													
	a	**39.65**	**1,77**	**<0.001**	2.47	1,77	0.12	-	-	-	-	-	-
	b	**18.87**	**1,76**	**<0.001**	**5.19**	**1,76**	**0.026**	**12.94**	**1,76**	**<0.001**	-	-	-
	c	3.2	1,76	0.078	0.96	1,76	0.331	-	-	-	**75.05**	**1,76**	**<0.001**
Total Dry Mass													
	a	**37.95**	**1,77**	**<0.001**	**4.12**	**1,77**	**0.046**	-	-	-	-	-	-
	b	**19.33**	**1,76**	**<0.001**	**6.1**	**1,76**	**0.016**	**6.74**	**1,76**	**0.011**	-	-	-
	c	0.83	1,76	0.364	**7.42**	**1,76**	**0.008**	-	-	-	**917.13**	**1,76**	**<0.001**

In search of genetic constraints limiting the evolution of egg size: Direct and correlated responses to artificial selection on a prenatal maternal effector

Pick, J.L., Hutter, P., & Tschirren, B

Supplementary Materials

S1 Relative and absolute egg size

Relative egg size was highly correlated with absolute egg size in all lines, replicates and generations (mean $r \pm$SD, 0.884 $\pm$ 0.120, n=14), We tested whether the strength of this correlation changed over the course of the experiment, by regressing correlation coefficients against generation. The correlation between absolute and relative egg size did not change over the course of the experiment ($F_{1,12} = 0.20$, $P = 0.661$; Figure S1).

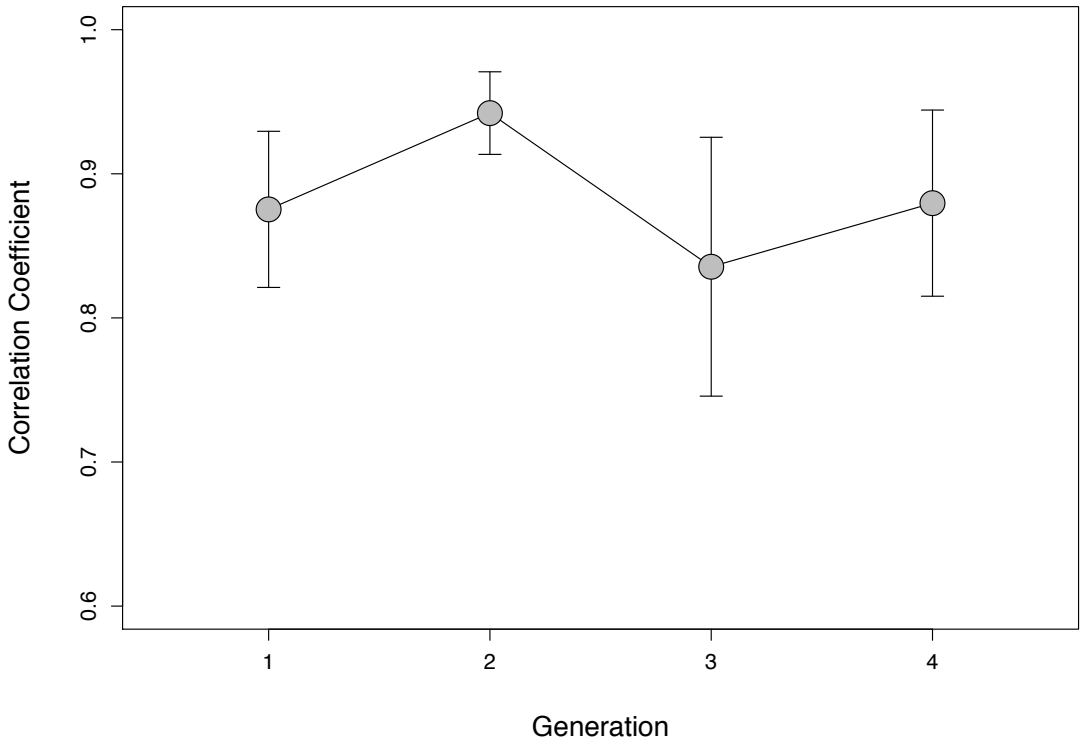


Figure S1: Correlation coefficients between relative and absolute egg size across generations.

S2 Unselected base population

Alongside the selection lines, an unselected base population was maintained in the same facility, originating from the same founder populations. These birds were not bred at the same times or ages as the selection lines and so are not directly comparable. Nevertheless, given that there was no directional change in mean egg size over 5 generations in this unselected population ($F_{1,3} = 0.51$, $P = 0.528$; see Figure S2), we can exclude the possibility that systematic changes in egg size have occurred over time due to inadvertent effects of husbandry.

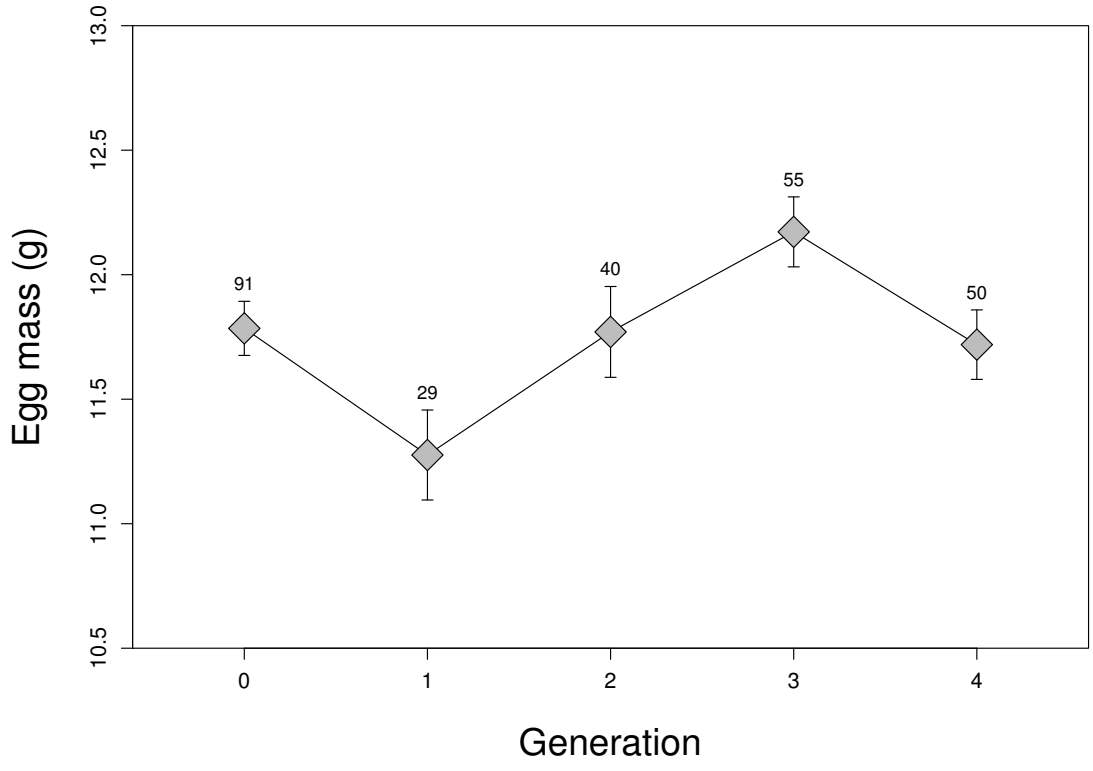


Figure S2: Egg size in the unselected base population across generations. Egg size fluctuates randomly between generations, but not in a directional manner across 5 generations. The number above each point represents sample size. Note that these females were bred at a different time to the two selection line replicates, and so cannot be used as a direct control.

S3 Changes in egg traits over the course of the laying sequence

We measured the repeatability of egg size and components in generation 4, using eggs collected on days 1, 2, 15 and 16 of egg collection (80 females, 2-4 eggs per female). Egg size and all egg components were highly repeatable within females (Table S1).

Table S1: Repeatabilities of egg traits of the dissected and dried eggs in generation 4 of the selection lines.

Trait	$r \pm$ SE	$F_{79,204}$	P
Egg Size	0.855 ± 0.024	21.92	< 0.001
Dry Mass	0.822 ± 0.029	17.38	< 0.001
Wet Albumen	0.879 ± 0.021	26.72	< 0.001
Dry Albumen	0.871 ± 0.022	24.91	< 0.001
Wet Yolk	0.801 ± 0.032	15.25	< 0.001
Dry Yolk	0.798 ± 0.032	15.06	< 0.001
Dry Shell	0.811 ± 0.031	16.23	< 0.001

In order to test whether egg traits change over the course of the laying sequence, we compared eggs laid at the beginning and end of the laying sequence using linear mixed effects models. Within-individual means of eggs measured at the beginning and end of the sequence were used in the analysis, and only individuals that had eggs measured at both time points were included in the analysis (n = 77 females). Timepoint, line and replicate were included as fixed factors, as well as the interaction between timepoint and line (indicating a line specific change in egg components). Female ID was included as a random effect. Significance was determined using likelihood ratio tests between nested models, meaning degrees of freedom in all comparisons was 1.

Dry albumen mass significantly increased over the laying sequence (Table S2, Figure S3). The effect of selection line on egg mass and wet albumen mass became

more pronounced later in the laying sequence (Table S2, Figure S3). Total dry mass, wet yolk, dry yolk and dry shell masses all did not change over the laying sequence (Table S2), although there was a tenancy for the selection line effect presented in the results to become more pronounced later in the laying sequence (Table S2).

Table S2: Difference between egg traits at the beginning and end of a laying sequence.

Trait	Line		Timepoint		Line x Timepoint		Replicate	
	χ^2	p	χ^2	p	χ^2	p	χ^2	p
Egg Size	-	-	-	-	**5.11**	**0.024**	1.15	0.283
Dry Mass	**26.10**	**<0.001**	0.13	0.716	3.45	0.063	3.47	0.062
Wet Albumen	-	-	-	-	**6.04**	**0.014**	0.00	0.956
Dry Albumen	**35.39**	**<0.001**	**8.10**	**0.004**	2.58	0.108	0.39	0.532
Wet Yolk	**12.54**	**<0.001**	0.12	0.726	2.97	0.085	**4.85**	**0.028**
Dry Yolk	**12.22**	**<0.001**	0.11	0.739	2.91	0.088	**4.11**	**0.043**
Dry Shell	**29.15**	**<0.001**	2.12	0.145	2.75	0.097	1.67	0.197

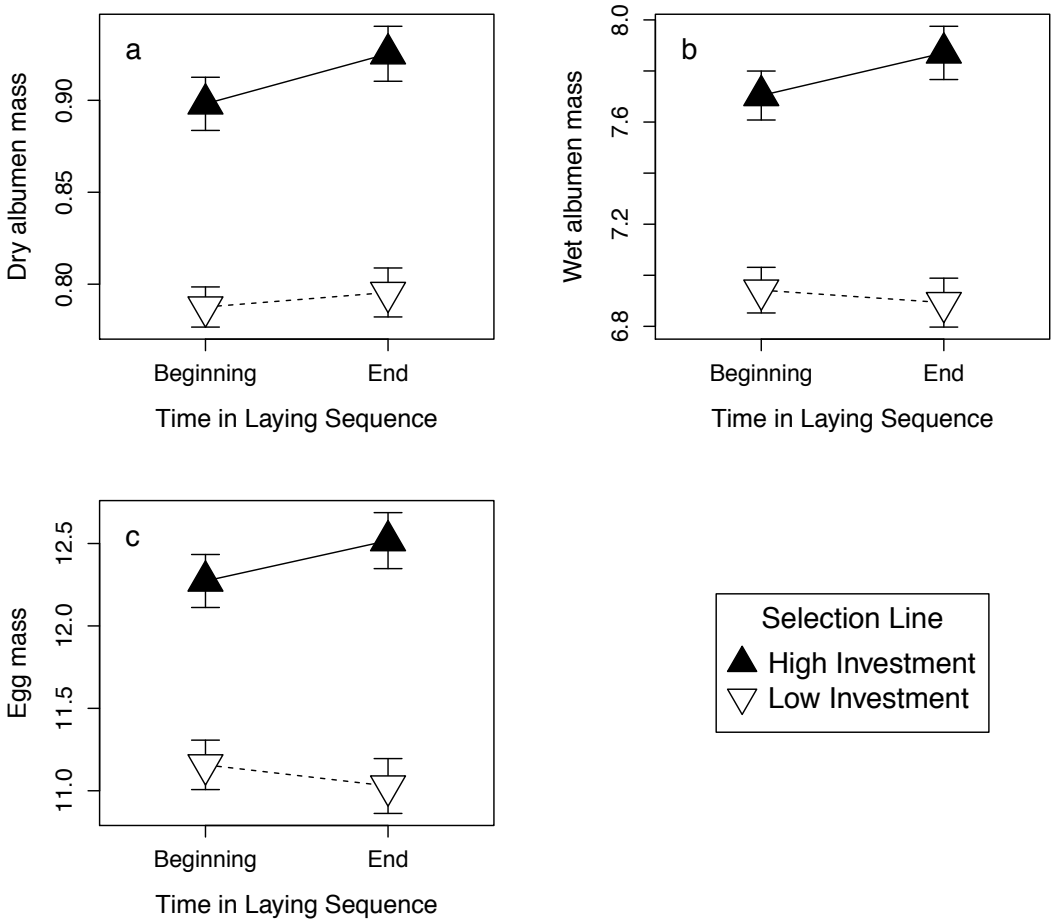


Figure S3: Difference between a) Dry Albumen Mass, b) Wet albumen mass and c) Egg mass at the beginning and end of a measured laying sequence.

S4 Difference in egg size between selection lines

Table S3: Difference in egg size between high and low investment lines across generations and replicates.

Generation	Replicate	Difference (g)	t	df	P
2	1	0.58	2.15	38	0.038
3	1	0.85	2.58	38	0.014
4	1	1.21	3.91	38	0.000
5	1	1.79	5.62	28	0.000
2	2	0.58	2.17	38	0.037
3	2	0.89	3.04	37	0.004
4	2	1.48	5.39	38	0.000
5	2	1.34	3.27	29	0.003